

Secreted Factors from Human Mast Cells Trigger Inflammatory Cytokine Production by Human Airway Smooth Muscle Cells

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Key Words

Airway inflammation • Asthma • Cytokines • Fc receptor (high-affinity IgE receptor) • Immunopharmacology • Mast cells • Human airway smooth muscle cells • Exosomes

Abstract

Background: A notable feature of allergic asthma is the infiltration of mast cells into smooth muscle in the human airway. Thus, mast cells and human airway smooth muscle (hASM) cells are likely to exhibit mutual functional modulation via direct cell-cell contact or through released factors. This study examined mast cell modulation of hASM cell cytokine release. **Methods:** The mast cell line HMC α was used to model mast cell function. hASM cells were either co-cultured directly with resting or IgE/antigen-stimulated HMC α cells or treated with HMC α -conditioned media to examine the impact on cytokine release. The activation pathways triggered in hASM cells by the mast cell-derived factors were examined through the use of selective inhibitors and by Western blotting. **Results:** HMC α cells, or their conditioned media, induced the expression of cytokines (IL-8 and IL-6) by hASM cells at both the mRNA and the protein level. Cytokine expression in hASM cells was greatly amplified when HMC α cells were IgE/antigen-activated. The effects of the conditioned media were not mediated by the chemokines MCP-1

and MIP-1 α or by exosomes. While the mast cell-derived factor(s) increased p38^{MAPK} phosphorylation in hASM cells, cytokine production was not inhibited by the p38^{MAPK} inhibitor SB203580. hASM cell production of IL-8 induced by HMC α condition media but not IL-6 was, however, attenuated by the Src tyrosine kinase inhibitor PP2. **Conclusions:** Our study shows that the release of soluble mediators by activated mast cells can stimulate hASM cells to elicit production of proinflammatory cytokines that may then exacerbate airway inflammation in asthma.

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Introduction

Mast cells and human airway smooth muscle (hASM) cells are central cell types in the pathobiology of asthma [1, 2]. Increased numerical density of mast cells in the airway smooth muscle bundles of human airways is a unique feature of asthma [3, 4]. This observation identifies mast cell/hASM cell interactions as a potentially important influence on the asthmatic phenotype.

The localization and adhesion of mast cells within airway smooth muscle bundles likely facilitates specific interactions between both cell types via direct cell-cell contact or through released factors that may modulate cell

function reciprocally. hASM cells have the capacity to produce many cytokines and chemokines, such as the C-X-C motif chemokines CXCL10 (IP-10), CXCL12, IL-8, and eotaxin [5–7]. These chemokines exhibit mast cell chemotactic activity through binding of their respective chemokine receptors [5, 8, 9]. After localization into the airway smooth muscle bundles, mast cells adhere to hASM cell avidly, with cell adhesion molecule 1 (CADM1) being important in this regard [10].

While hASM cell contact has been shown to enhance mast cell degranulation [11, 12], direct interaction between these cell types can also modulate hASM cell activity. hASM cells co-cultured with mast cells, or mast cell-derived membranes, showed elevated eotaxin production, which was greater when cells were in direct contact [13].

In addition to direct cell-cell contact, hASM cells and mast cells could also communicate by releasing soluble mediators [14, 15]. Mast cell mediators such as histamine, cysteinyl leukotrienes, and prostaglandin D₂ can cause airway contraction, while tryptase, chymase, TNF- α , TGF- β , and PDGF can stimulate hASM cell proliferation and the deposition of ECM proteins, contributing to airway remodeling [16–20]. Moreover, IL-1 β , TNF- α , and TGF- β can enhance the release of cytokines (e.g. IL-8, IL-6, and eotaxin) from hASM cells [7, 21–23]. However, in keeping with their emerging role as negative as well as positive regulators of inflammation [24], mast cells are also capable of secreting IL-4, IL-10, and IL-13 [25], and treatment of hASM cells with these cytokines has been shown to inhibit IL-8 and RANTES secretion [7].

Mast cells are also known to release exosomes. These small lipid vesicles are known to contain a number of immunoregulatory proteins such as MHC and tetraspanins as well as mRNA and microRNAs [26, 27]. These molecules can be presented/transferred to recipient cells, thus regulating cellular processes such as lymphocyte proliferation and mediator release from endothelial cells [26, 28–30]. However, the importance of mast cell-derived exosomes for hASM cell activity has not been investigated.

We have examined the actions of mast cells on hASM cell function by co-culturing these cell types to model the cell behavior occurring in the airway environment which is influenced by a complex mix of factors. In contrast to earlier studies [13, 31], the HMC α cell line, generated by stable transfection of HMC-1 cells with the α -subunit of human Fc ϵ RI (HMC α cells) [32], was utilized throughout to also permit investigation of IgE-dependent activation of the mast cells.

Methods and Materials

Cell Culture

hASM was dissected from bronchi obtained during lung resection surgery (kindly provided by The Alfred Hospital, Melbourne, Vic., Australia) as approved by The University of Melbourne Human Ethics Committee. hASM cells were prepared as described previously [33] and then maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with heat-inactivated fetal bovine serum (FBS; 10% v/v), L-glutamine (2 mM), penicillin G (100 U/ml), streptomycin (100 μ g/ml) (all from Invitrogen), HEPES (16 mM), sodium bicarbonate (0.2% v/v), and nonessential amino acids (1% v/v) (all from Sigma). Cells at passage numbers between 3 and 15 were used. Nine separate specimens were obtained for this study from 1 healthy donor and 8 donors with respiratory disorders (age 14–63 years; 4 male, 4 female, 1 information not available). The indications for resection/diagnoses of the donors were: asthma (n = 2), pulmonary hypertension (n = 1), bronchiectasis (n = 1), lymphangioleiomyoma (n = 1), pulmonary fibrosis (n = 1) and pulmonary emphysema (n = 1). Information regarding diagnoses was not available for one of the donors. Telomerase-immortalized hASM cell lines [34] (kindly provided by Prof. William Gerthoffer, University of South Alabama, Mobile, Ala., USA) were cultured identically to the primary hASM cells. hASM cells were, unless stated, serum starved for between 24 and 48 h (medium as above but with 0.25% w/v BSA replacing the FBS) before use. Monomed A (SAFC Biosciences), comprising insulin, transferrin, and selenium, was added to all serum-starved cells (1% v/v) during this period.

Human mast cell line (HMC-1) cells [35] (kindly provided by Prof. Joseph Butterfield, Mayo Clinic, Rochester, Minn., USA) transfected with the human Fc ϵ RI α -subunit (HMC α) [32] were cultured in Iscove's modified Dulbecco's medium (IMDM; HyClone), supplemented with FBS (10% v/v), glutamine (2.0 mM), penicillin (500 U/ml), streptomycin (500 μ g/ml), G418 (125 μ g/ml) (all from Invitrogen), and alpha-thioglycerol (0.01% v/v; Sigma).

Generation of HMC α Cell-Derived Conditioned Medium

HMC α cells were incubated with human IgE (hIgE) [anti-NP (clone JW8 (ECACC); 1 μ g/ml)] for 3 days, and then incubated in serum-free DMEM after washing once with PBS. The cells (1 $\times$ 10⁶ cells/ml) were treated with or without antigen (NIP-BSA; 3 ng/ml) for 4 h, and the HMC α cell-derived conditioned medium (CM) was harvested by centrifugation of supernatants (200 g, 5 min), followed by filtration through a 0.2- μ m porous membrane.

Ultracentrifugation of CM for Exosome Depletion

A differential ultracentrifugation protocol was used to remove exosomes from the HMC α cell-derived CM [36]. Briefly, HMC α cell-derived CM was harvested as described above and then transferred to a 10-ml polycarbonate ultracentrifuge tube (Thermo Scientific) and centrifuged (10,000 g, 30 min, 4°C) to remove further cell debris. The supernatants were then transferred to a fresh tube and centrifuged (100,000 g, 70 min, 4°C) to sediment exosomes. Both centrifugation steps used a Sorvall WX Ultra90 centrifuge (Thermo Scientific). The exosome-depleted supernatants were then carefully removed and used immediately to treat hASM cells.

Cell Stimulation and Cytokine ELISA

hASM cells were plated in 12-well tissue culture plates (1×10^5 cells/well). The cells were allowed to attach to the wells over approximately 15 h, and they were then serum starved for a further 24 h. HMC α cells were sensitized with hIgE for 3 days in serum-complete IMDM. hASM and HMC α cells cultured alone were used as controls. To examine the functional effects of direct cell-cell contact, HMC α cells were washed with PBS and resuspended in serum-free DMEM and then added to the wells containing hASM cells. HMC α cells were also used to generate the CM as described above. Both the nonstimulated and the antigen-stimulated HMC α cell-derived CM were then added to the serum-starved hASM cells to examine the effects of HMC α -derived soluble factor(s) on hASM cell cytokine production.

In most situations, cells were co-cultured at an hASM cell/mast cell ratio of 1:10. A 4:1 hASM cell/mast cell ratio was also studied, however, as this better reflects the ratio reported in vivo [11]. The cell-cell co-cultures, hASM or HMC α cell alone groups were then treated with or without antigen (3 ng/ml) for 24 h. In studies to determine the heat-sensitivity of released factors, antigen-stimulated HMC α cell CM was heated to various temperatures (21–99°C) for 30 min, diluted 1/50 in serum-free DMEM, and added to hASM cells for 24 h. In some experiments hASM cells were stimulated with various concentrations of either MIP-1 β or MCP-1 (ProSpec) for 24 h.

Pathways potentially involved in the intracellular signaling underlying the activity of the HMC α cell-derived CM were examined using the Src kinase inhibitor PP2, the p38^{MAPK} inhibitor SB203580, the Jun N-terminal kinase (JNK) inhibitor SP600125 (all from Enzo Life Sciences), and the MAP kinase 1 and 2 (MEK 1 and 2) inhibitor U0126 (Tocris Bioscience), all of which were added to hASM cells 30 min prior to addition of the HMC α cell-derived CM. The glucocorticoid dexamethasone was also used as a control agent known to inhibit cytokine production from hASM cells. In all cases, hASM cell supernatants were then harvested and assayed for IL-8 and IL-6 levels using commercially available ELISA kits (OptEIA; BD Biosciences) according to the manufacturers' instructions, with absorbance measured using a plate reader (Multiskan Ascent; Thermo Scientific) and cytokine concentrations determined from standard curves using Ascent software version 2.6 (Thermo Scientific).

Quantitative Real-Time PCR Detection of Cytokine mRNA

hASM cells were treated with HMC α cell-derived CM for 6 or 16 h. The mRNA was extracted from cells using an RNeasy Mini Kit according to the manufacturer's protocol (Qiagen). mRNA was quantified and purity assessed using a NanoDropTM 2000 (Thermo Scientific). The purified total RNA was used as a template to generate first-strand cDNA via reverse transcription. The reaction mix contained RNA (0.5 μ g), Superscript III reverse transcriptase (20 U; Invitrogen), random hexamers (37.5 ng; Promega), 2-deoxynucleotide 5'-triphosphate (dNTP; 0.5 mM) mix (Promega), and RNase inhibitor (2 U; Invitrogen) in a final volume of 5 μ l. Reverse transcription was performed with a thermocycler (GeneAmp 2400; Perkin-Elmer) using the following conditions: 25°C/10 min, 37°C/60 min, and 95°C/5 min. The resulting cDNA was diluted 1:40 with sterile water and stored at –20°C until use.

An ABI Prism 7900HT sequence detection system (Applied Biosystems) was used to quantitatively analyze the level of IL-8 or

IL-6 gene expression in the samples being tested. Each real-time reaction (10 μ l; conducted in a 384-well plate) contained diluted cDNA (3 μ l), Platinum SYBR Green qPCR Supermix-UDG (5 μ l; Invitrogen), and gene-specific forward and reverse primers (0.2 μ M; Invitrogen). 18S ribosomal RNA (18S rRNA) was used as a housekeeping gene. Primer sequences used were IL-8F; 5'-CTG GCC GTG GCT CTC TTG-3', IL-8R; 5'-CCT TGG CAA AAC TGC ACC TT-3', IL-6F; 5'-AGC TCT ATC TCG CCT CCA GGA-3', IL-6R; 5'-CGC TTG TGG AGA AGG AGT TCA-3', 18S rRNAF; 5'-CGC CGC TAG AGG TGA AAT TC-3', 18S rRNAR, and 5'-TTG GCA AAT GCT TTC GCT C-3'. Real-time PCR reactions were carried out in triplicate using the following cycle conditions: 50°C/2 min, and 95°C/10 min to activate the DNA polymerase, which was then followed by 40 cycles of denaturation at 95°C/15 s and annealing/extension at 60°C/60 s. The software SDS 2.1 (Applied Biosystems) was used to generate threshold cycle (Ct) values.

The mRNA levels of IL-8 and IL-6 were expressed using the $\Delta\Delta$ Ct and relative value method. Firstly, Δ Ct values were generated by subtracting the Ct value for the target gene from the Ct value of the 18S rRNA. $\Delta\Delta$ Ct values were then generated by subtracting the Δ Ct value of the treatment group from the Δ Ct value of the control group. The relative fold expression of the target genes was then calculated as $2^{-\Delta\Delta$ Ct}.

Western Blot Analysis

hASM cells were plated in 6-well plates (2×10^5 cells/well). The cells were allowed to attach over 15 h and were then serum starved for 24 h. Cells were then treated with HMC α cell-derived CM for 15 min or 1 h. Cells were washed with ice-cold PBS ($\times 2$), and then lysed with lysis buffer [1% v/v Brij-96, 150 mM NaCl, 10 mM Tris-HCl (pH 7.5)], containing sodium orthovanadate (1 mM) and a protease inhibitor cocktail (Sigma). The lysate was collected and clarified by centrifugation (13,000 g, 15 min, 4°C). Lysates were denatured and reduced in SDS sample buffer (0.05 M Tris-HCl, 0.1% w/v SDS, 10% v/v glycerol, 50 mM dithiothreitol), and separated by 10% SDS-polyacrylamide gel electrophoresis, and resolved proteins were transferred onto PVDF membranes (GE Healthcare). The membranes were blocked [2% (w/v) BSA in TBS/0.05% Tween 20] for 1 h and then probed with either anti-phospho-ERK1/2 (Thr202/Tyr204) or anti-phospho-p38^{MAPK} (Thr180/Tyr182) (Cell Signaling Technology) overnight at 4°C. Subsequently, membranes were washed in TBS with 0.05% Tween 20 and incubated with the appropriate HRP-conjugated secondary antibodies (Chemicon) at room temperature for 2 h. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL; GE Healthcare) using an ImageQuant 350 imager (GE Healthcare). Blots were then stripped (1.5% w/v glycine, 0.1% w/v SDS, 1% v/v Tween 20, pH = 2.2) and reprobed with either anti-p38^{MAPK} or anti-ERK1/2 antibodies (Santa Cruz) and visualized again. Band intensities were quantified by densitometry using the Image J program (National Institutes of Health, Bethesda, Md., USA). The MAPK phosphorylation levels were normalized to total MAPK, and the data were then expressed as percentages of control levels at the 15-min time point.

Statistical Analysis

Data from ELISA and Western blot analysis are expressed as means $\pm$ SEM, where n represents the number of independent primary hASM cell cultures used or the number of experiments

repeated when using hASM cell lines. For ELISA, Western blot, and qPCR data, differences between treatment groups were tested using one-way repeated measures ANOVA or two-way repeated measures ANOVA, followed by Bonferroni's post hoc test for multiple comparisons. A two-tailed paired Student's t test was also used to compare certain groups of ELISA data. $p < 0.05$ was considered statistically significant. Statistical analysis was performed using GraphPad Prism® for Windows (version 5.00; GraphPad Software).

Results

HMC α Cell-Derived CM Elicits Cytokine Release from hASM Cells

In keeping with our previous findings, IgE-sensitized HMC α cells basally released no detectable IL-8 or IL-6 but, following challenge with antigen, significant secretion of these cytokines was observed (fig. 1a) [32]. hASM cells basally secrete IL-8 and IL-6 and the cells produced similar levels of the measured cytokine after treatment with hIgE/antigen (fig. 1a), consistent with previous findings that there is no functional IgE receptor expression on cultured hASM cells [31, 37].

hASM cells co-cultured with HMC α cells produced greater levels of IL-8 and IL-6 compared to when hASM cells were cultured alone. Similarly, transfer of the non-stimulated HMC α cell-derived CM to hASM cells also induced IL-8 and IL-6 production (fig. 1a).

hASM cells co-cultured with HMC α cells, and then challenged with antigen, showed a large enhancement of the production of all measured cytokines which was significantly greater than the cytokine release from hASM cells cultured alone and with non-antigen-activated HMC α cells. CM derived from antigen-activated HMC α cells had a similar effect (fig. 1a). These results demonstrate that HMC α cells produce soluble factor(s) with the capacity to trigger significant cytokine release from hASM cells, with this ability being enhanced by antigen-activation of HMC α cells. It is noteworthy that the quantities of IL-8 and IL-6 released from the CM-activated hASM cells is several orders of magnitude greater than the levels of these cytokines released directly from antigen-activated HMC α cells. This places the hASM cell as the dominant producer of IL-8 and IL-6 in these studies.

A co-culturing ratio of 4:1 hASM cells/HMC α cells was also studied to better reflect the cell ratio occurring in vivo [11]. Essentially similar results to those obtained above were generated, albeit with reduced quantities of cytokines liberated (fig. 1b).

Consistent with the released factors inducing transcriptional activation of IL-8 and IL-6 genes, HMC α CM produced a trend towards an increase in the expression levels of mRNA for these cytokines, with this effect becoming statistically significant upon stimulation of HMC α cells with hIgE/antigen (online suppl. fig. 1; see www.karger.com/doi/10.1159/000339697 for all online suppl. material).

Exosome Depletion of HMC α Cell-Derived CM Does Not Reduce Its Stimulatory Effects on IL-8 Production

Previous studies have shown that HMC-1 cells can release exosomes with a range of biological actions [28, 30]. We therefore sought to determine whether these small vesicles were responsible for the bioactivity in the HMC α cell supernatants.

The experiment was performed as described above, with one extra condition added in which exosomes were depleted from the HMC α CM by ultracentrifugation. In keeping with results in figure 1, transfer of the HMC α CM significantly induced IL-8 production from hASM cells, with this being enhanced by hIgE/antigen stimulation of the HMC α cells. However, centrifugal depletion of exosomes had no significant effect on the ability of the HMC α CM to induce IL-8 release from hASM cells. There was, however, a trend towards a decrease in hASM cell IL-8 production following exosome depletion from the hIgE/antigen-stimulated HMC α cell-derived CM (fig. 2).

HMC α Cell-Derived Active Factor(s) Are Heat Labile

To test the heat stability of the active factor(s), the undiluted antigen-stimulated HMC α CM was heated to various temperatures (21–99°C) for 30 min, diluted 50-fold with fresh serum-depleted DMEM, and added to hASM cells. Incubation of HMC α CM at 21–60°C had little effect on its ability to induce IL-8 or IL-6 release from hASM cells. However, the HMC α CM started to lose functional activity at 70°C and was significantly reduced at 80°C and 90°C (online suppl. fig. 2). This heat sensitivity, combined with membrane filtration data that showed that the factor had a molecular mass >10 kDa (data not shown), provides evidence that the mast cell-derived active factor is likely a proteinaceous mediator.

Treatment of hASM Cells with MIP-1 β or MCP-1 Does Not Induce Cytokine Release

We have previously profiled the cytokines released from IgE/antigen-stimulated HMC α cells using a 17-plex array [32]. Among the released cytokines, MIP-1 β and MCP-1 were, in terms of quantity, the two predominant

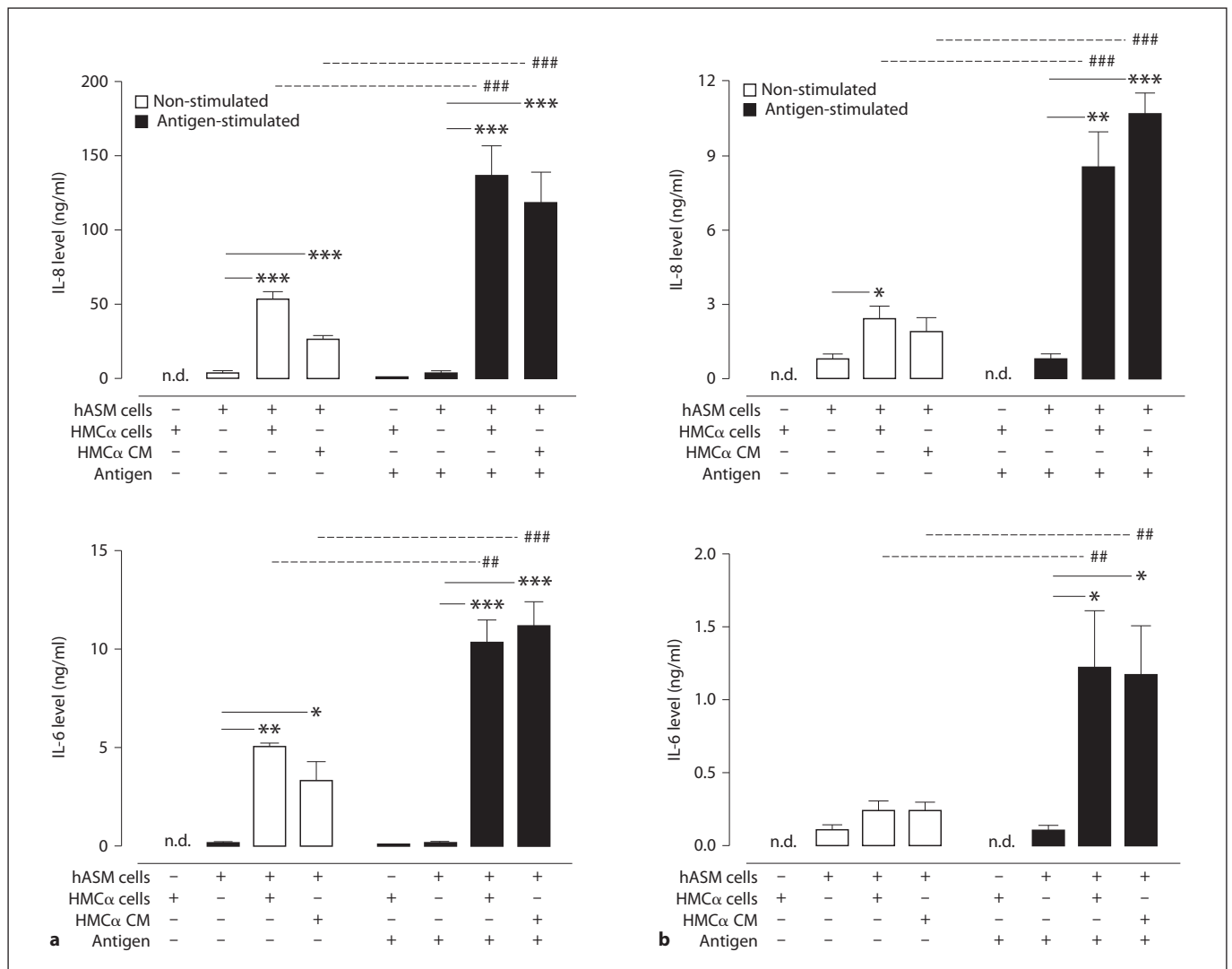


Fig. 1. Co-culture of HMCα cells and hASM cells, or addition of HMCα cell-derived CM, triggers the release of large quantities of cytokines from hASM cells. Co-culturing ratios of 1:10 (**a**) and 4:1 (**b**) hASM cell/mast cell were examined. IL-8 and IL-6 were measured after 24 h of stimulation of hASM cells with/without HMCα cell-derived CM or co-culturing with HMCα cells. HMCα cells were either nonstimulated or activated through FcεRI by hIgE/antigen stimulation. Results are expressed as the mean cytokine levels $\pm$ SEM of experiments conducted on 3–9

different hASM cell cultures. One-way repeated measures ANOVA, followed by Bonferroni's multiple post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparing hASM cell and HMCα cell co-cultures or hASM cell and HMCα CM to hASM cell alone cultures. Two-way repeated measures ANOVA, followed by Bonferroni's multiple post hoc test, was applied for comparisons of the antigen-stimulated group to the nonstimulated group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. n.d. = Not detected.

cytokines produced. Furthermore, the relatively high basal release of MCP-1 could potentially account for the ability of the nonstimulated HMCα cell-derived CM to activate hASM cells. However, MCP-1, MIP-1β, or a combination of the two chemokines did not produce a significant change in IL-8 release from hASM cells (data not shown).

HMCα Cell-Derived CM Induces p38^{MAPK} Phosphorylation in hASM Cells

MAPK pathways have been well studied in relation to their regulation of cytokine production in hASM cells [13, 38–42]. We examined whether treatment of hASM cells with HMCα CM affected the activation of ERK1/2 and p38^{MAPK}. HMCα CM induced p38^{MAPK}, but not

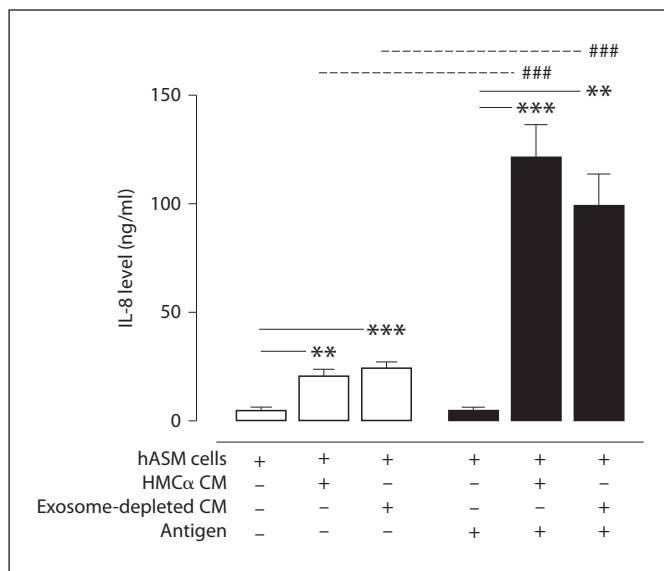


Fig. 2. Depletion of exosomes from HMC α cell-derived CM does not affect CM induction of IL-8 production from hASM cells. Results are expressed as the mean cytokine level $\pm$ SEM of experiments conducted on 3 different hASM cell cultures. ** $p < 0.01$, *** $p < 0.001$ as compared to the hASM cells alone culture; one-way repeated measures ANOVA. ### $p < 0.001$ comparing the hIgE/antigen-stimulated group to the nonstimulated group; two-way repeated measures ANOVA, followed by Bonferroni's multiple post hoc test.

ERK1/2, activation after 15 min. Sustained phosphorylation, albeit to a lesser extent, was also observed 1 h after stimulation (fig. 3). In keeping with the increased levels of bioactivity released from HMC α cells following hIgE/antigen-stimulation, the p38^{MAPK} phosphorylation was also enhanced by antigen activation of hASM cells (fig. 3).

HMC α CM-Mediated IL-8 Production Is Inhibited by Src Kinase Inhibition, but Not MAPK Pathway Inhibition

We next assessed whether the activation of p38^{MAPK} directly contributes to the HMC α CM-mediated cytokine production from hASM cells. While hASM cell treatment with the glucocorticoid dexamethasone (100 nM) produced strong inhibition of cytokine release across all conditions tested, pretreatment of hASM cells with the p38^{MAPK} inhibitor (SB203580; 1 μ M) or inhibitors of JNK (SP600125; 1 μ M) and ERK1/2 (U0126; 1 μ M) did not inhibit either resting or hIgE/antigen-stimulated HMC α CM-mediated cytokine (IL-8 and IL-6) production. However, CM-induced release of IL-8, but not IL-6, was inhibited by the Src kinase inhibitor PP2 (10 μ M; fig. 4).

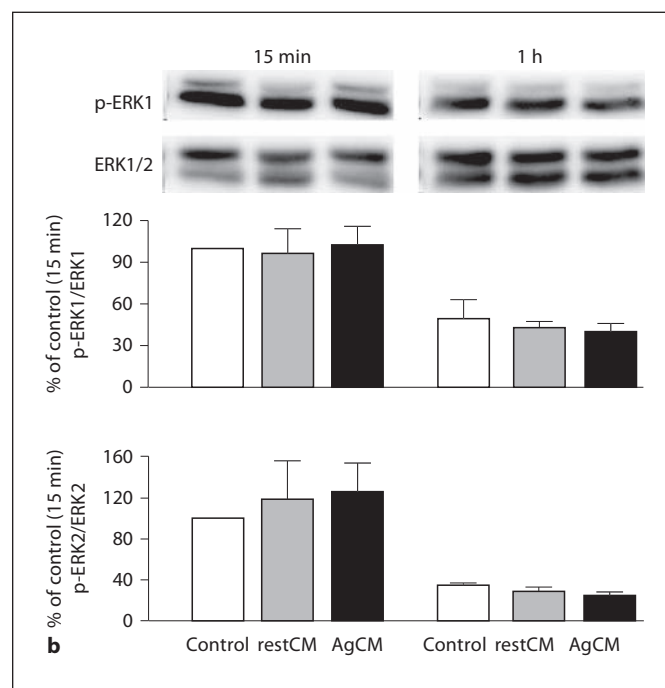
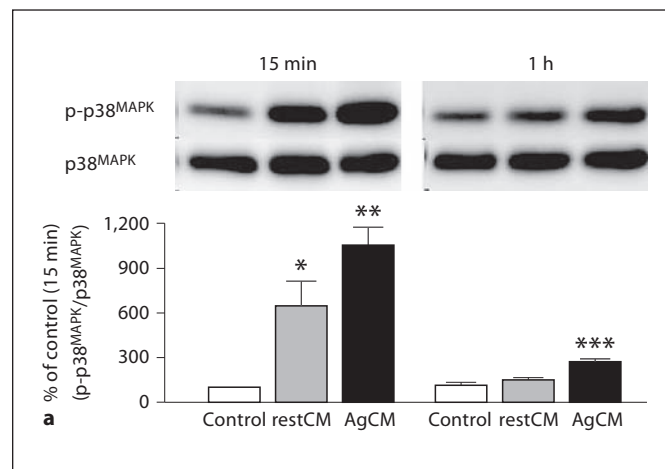
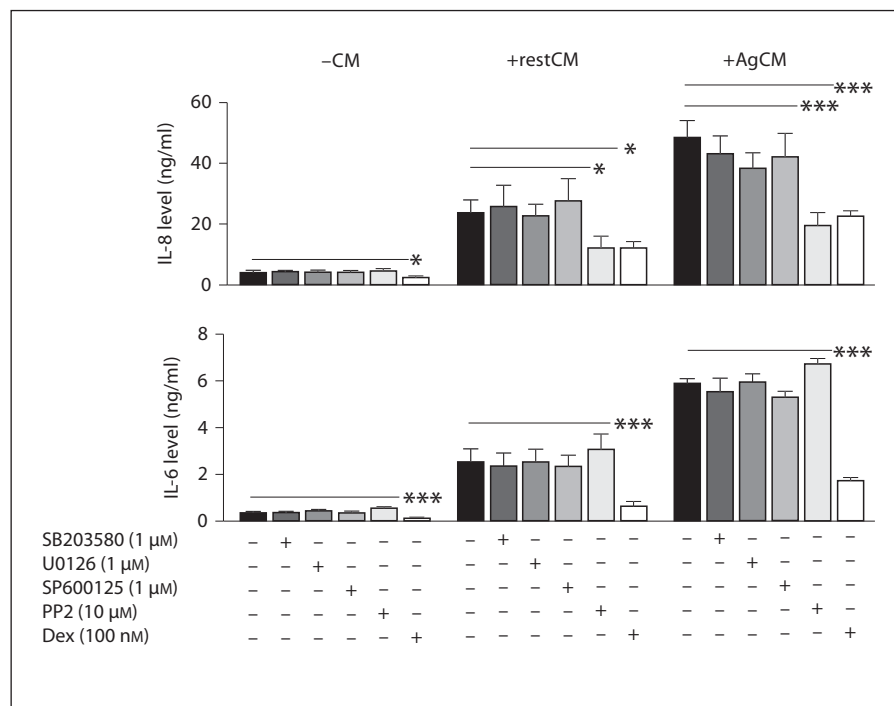


Fig. 3. Treatment of hASM cells with HMC α cell-derived CM leads to p38^{MAPK} (a) but not ERK1/2 (b) phosphorylation. hASM cells were serum starved for 24 h and then treated with either non-stimulated HMC α cell CM (restCM) or antigen-stimulated HMC α cell CM (AgCM) for either 15 min or 1 h prior to cells being lysed and examined by Western blotting using a phospho-p38^{MAPK} or phospho-ERK1/2 specific antibody and total anti-p38^{MAPK} and ERK1/2 antibodies. Blots shown are representative of experiments conducted on 3 separate experiments using 1 immortalized hASM cell line. The band intensities were quantified by densitometry using the Image J program. The MAPK phosphorylation levels were determined by normalization of phosphorylated MAPK over total MAPK intensities and are expressed as the mean percentage of the 15-min control level $\pm$ SEM. Two-way repeated measures ANOVA followed by Bonferroni's post hoc test was applied for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the control group.

Fig. 4. Inhibition of the HMC α CM-mediated IL-8 and IL-6 production from hASM cells by the Src kinase inhibitor PP2 and glucocorticoid dexamethasone. hASM cells were serum starved for 24 h and preincubated with the p38^{MAPK} inhibitor SB203580, the JNK inhibitor SP600125, the ERK1/2 inhibitor U0126, Src kinase inhibitor PP2, or dexamethasone for 30 min prior to activation of cells with either non-stimulated HMC α cells CM (restCM) or antigen-stimulated HMC α cells CM (AgCM) (1:10 dilution) for 24 h. Levels of IL-8 and IL-6 were then measured by immunoassay. Drug diluent (DMSO 0.1%) had no measurable effect on IL-8 release. Results shown are means $\pm$ SEM of 4 separate experiments using 1 immortalized hASM cell line. One-way repeated measures ANOVA followed by Bonferroni's post hoc test was applied for multiple comparisons. * $p < 0.05$, *** $p < 0.001$ for comparison to no-inhibitor groups.



Discussion

A distinguishing feature of allergic asthma is the infiltration of mast cells into human airway smooth muscle bundles, which is not observed in other related airway disorders [3, 4]. Previous studies have reported that hASM cells are able to release a number of chemokines to recruit mast cells into the smooth muscle bundles in human airways [5, 11, 43, 44]. Recruited mast cells can then firmly adhere to the hASM cells partially via the adhesion molecule CADM1 [10] and extracellular matrix protein receptors CD51 and CD44 [45]. Thus, mast cells and hASM cells likely exhibit mutual functional modulation via direct cell-cell contact or through released factors that can potentially enhance and perpetuate asthmatic symptoms. The present study aimed to extend our understanding of the functional consequences of mast cell and hASM cell interactions. As previous studies have shown that coculture of mast cells and hASM cells did not affect proliferation of nonasthmatic hASM cells [31, 46], hASM cell cytokine production was focused upon in the present study.

Our results demonstrate that contact with HMC α cells induced cytokine release from hASM cells that was greatly enhanced by IgE/antigen stimulation of the HMC α cells. These results are in accordance with an ear-

lier study that showed enhanced eotaxin release after coculture of hASM cells and HMC-1 cells [13]. The actions of HMC α -derived CM were examined in parallel studies. CM derived from resting HMC α cells also triggered IL-8 and IL-6 production from hASM cells, but to a lesser extent than direct cell-cell contact. This observation emphasizes the importance, under resting conditions, of cell-cell contact for cell stimulation, as was shown previously using HMC-1 cells [13]. However, antigen-stimulated HMC α cell-derived CM had an effect similar to that of direct cell-cell contact, demonstrating a dominant role for a soluble factor released from the antigen-stimulated HMC α cells. The level of cytokine released by hASM cells in response to the antigen-stimulated HMC α cell-derived CM is of a significant biological magnitude. This effect might be expected to be further enhanced in the remodeled asthmatic airway where extensive smooth muscle hypertrophy and hyperplasia are known to occur [14, 15].

It is possible that the CM prevents degradation of the constitutively released cytokines from hASM, as cytokines such as IL-8, IL-6, and IL-13 are known to be degraded by mast cell proteases (chymase and cathepsin G) [47]. We thus examined whether CM derived from HMC α cells triggered cytokine production through driving cytokine gene transcription. We showed that the HMC α

cell-derived CM enhanced hASM cell IL-8 and IL-6 mRNA levels. However, the released factors in the CM might also act to enhance cytokine mRNA stabilization, a mechanism previously shown to be important in hASM cell cytokine release [48, 49].

A previous study showed that CM derived from activated human lung mast cells also increased IL-8 secretion but reduced eotaxin production from hASM cells [31]. Using HMC-1 cells, both we (data not shown) and others [13] have shown an enhancement of eotaxin release upon mast cell/hASM cell co-culture. This inconsistency is likely related to the different mast cells utilized between these studies. Unlike HMC-1 cells, primary lung mast cells have a more mature phenotype with abundant cytoplasmic granules that can undergo exocytosis, releasing granular mediators such as histamine and proteases such as tryptase. As we reported recently [32], HMC α cells do not degranulate to IgE/antigen activation, as measured through β -hexosaminidase release. Thus, there will be a different spectrum of mediators released from these different mast cells. This would be expected to have both functional and experimental implications. For instance, it has been shown that tryptase can cleave eotaxin [50], possibly accounting for the inconsistencies raised above. Thus, HMC α cells may be a good model system of the immature mast cells known to exist in the lung [51]; these cells perhaps represent newly recruited blood-borne mast cell progenitors. In addition, it is known that a subpopulation of lung mast cells, despite expressing Fc ϵ RI, show relatively modest degranulation responses [52].

Our data demonstrate that mast cell-derived soluble factor(s) can trigger cytokine production from hASM cells. It is known that mast cells release numerous mediators and cytokines (such as histamine, leukotrienes, tryptase, IL-1 β , and TNF α), which are capable of regulating hASM activities, such as contraction, cell proliferation, and triggering of cytokine production. The heat denaturation and molecular sizing profile of the CM suggested that the responsible factor(s) were likely proteinaceous. In a previous bioplex-cytokine assay, we examined the release of 17 inflammatory cytokines from HMC α cells [32]. MCP-1 and MIP-1 β were the two most notable chemokines released from HMC α cells, with MCP-1, by nature of its basal release from HMC α cells and the bioactivity of resting HMC α CM, being of particular interest. While a recent study has examined the actions of MIP-1 α on hASM cell proliferation [53], the effects of MCP-1 and MIP-1 β on hASM cell cytokine production have not been reported. Recombinant MCP-1, MIP-1 β , or a combination of the two chemokines did not

induce IL-8 production from hASM cells. This observation suggests that these chemokines are not the bioactive factors released from HMC α cells, or that other factors in the HMC α cell-derived CM might also be required. The bioplex assay showed that HMC α cells were also able to release a number of other cytokines, such as TNF α , IL-1 β , IFN γ , and IL-17, which are known to trigger cytokine production from hASM cells [7, 54]. However, based on the published activity of these cytokines on hASM cells [7, 54–56], the quantities released from HMC α cells are unlikely, as individual stimuli, to directly induce the level of IL-8 production from hASM cells that is mediated by the HMC α cell-derived CM. However, we cannot discount the cumulative, and possibly synergistic, actions of these cytokines accounting for the effects observed.

Recent studies reported that mast cells are able to communicate with other cells via the release of exosomes. These vesicles contain various immunoregulatory proteins as well as mRNAs and microRNAs [27, 57, 58]. Evidence has shown that mast cell-derived exosomes are able to induce lymphocyte proliferation and cytokine production [30, 59], and can also induce mediator release from endothelial cells [28]. We found that depletion of exosomes through ultracentrifugation did not significantly alter HMC α cell-derived CM-mediated IL-8 production from hASM cells. However, there was a trend towards reduction of the stimulatory response when exosomes were depleted from the antigen-stimulated HMC α cell-derived CM. This result suggests that exosomes might partially contribute to mast cell-mediated hASM cell modulation, but it is clear that soluble factors are critical to the effect.

MAPK pathways are well known to contribute to cytokine production by hASM cells, with the importance of the pathways being very much dependent upon the cytokine measured and the nature of the stimulus used [13, 38–42]. Phosphorylation of p38^{MAPK}, but not ERK1/2, was observed after treatment of hASM cells with HMC α cell-derived CM, which is consistent with a previous study that also showed phosphorylation of p38^{MAPK} after culturing hASM cells with HMC-1 cells [13]. However, the enhanced IL-8 and IL-6 production was not inhibited by a p38^{MAPK} inhibitor and also not by the inhibitors of other MAPKs (ERK1/2 and JNK). This observation suggests that MAPK pathways are apparently less functionally important for HMC α cell-derived CM-mediated cytokine production from hASM cells. Interestingly, however, IL-8 production was diminished by the Src kinase inhibitor PP2. This finding suggests that the HMC α cell-derived factor/factors act via a tyrosine kinase receptor or

perhaps a G protein-coupled receptor-mediated pathway involving an Src kinase.

We recently showed expression of the Src kinase Lyn in hASM cells [37]. Another study also reported Lyn expression in hASM cells alongside a number of other Src kinases including Fyn, YES, and c-SRC [60]. Co-stimulation of hASM cells with IL-1 β , TNF α , and INF γ has been shown to induce Src family tyrosine kinase activation which, when inhibited by the Src kinase inhibitor PP1, led to attenuation of IL-1 β , IL-6, and IL-8 release [61]. In our studies, the Src kinase inhibitor PP2 also diminished CM-induced IL-8 release from hASM cells but was, however, unable to inhibit the concomitant IL-6 release. This argues against HMC α -derived IL-1 β , TNF α , and INF γ being responsible for the hASM cell activation. It may be that there are multiple mast cell-derived factors differentially regulating IL-8 and IL-6 production from hASM cells or, alternatively, that there are different signaling pathways differentially controlling IL-8 and IL-6 production in hASM cells [37]. Src kinases are known to activate numerous signaling cascades [62], and further studies are thus required to reveal the pathway important for the ability of the HMC α -derived active factor/factors to induce IL-8 production from hASM cells.

In summary, the present study demonstrates that mast cell-derived proteinaceous factor/factors produce dramatic proinflammatory cytokine release from hASM cells through a signaling mechanism involving a Src kinase pathway. The infiltration of mast cells into ASM bundles in asthmatic subjects, and consequent cellular cross-talk, especially in the presence of allergen, could trigger large quantities of cytokine production from hASM cells exacerbating airway inflammation and recruiting further inflammatory cells that perpetuate asthmatic symptoms.

Acknowledgements

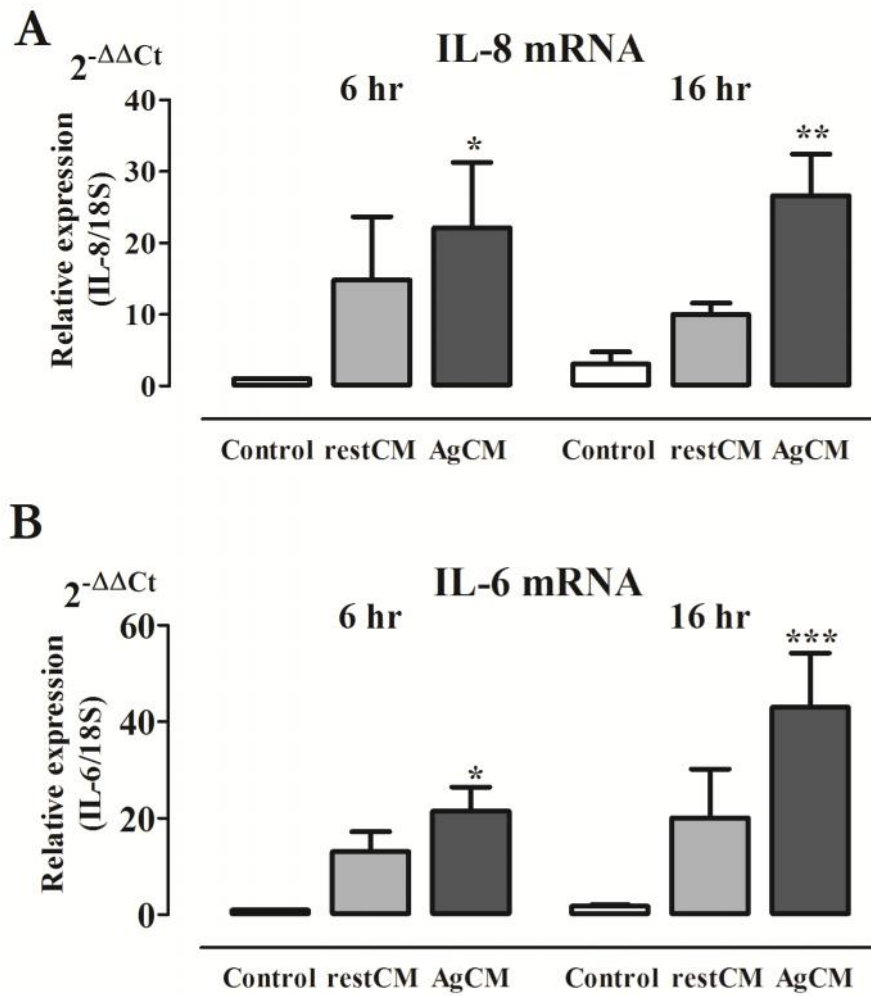
We thank Prof. Joseph Butterfield (Mayo Clinic, Rochester, Minn., USA) for providing the HMC-1 cells, and Prof. William Gerthoffer (University of South Alabama, Mobile, Ala., USA) for providing the telomerase-immortalized human airway smooth muscle cell lines. This study was partly supported by funding from The Asthma Foundation of Queensland, Australia (G.A.M.), and by the NH&MRC, Australia (A.G.S.).

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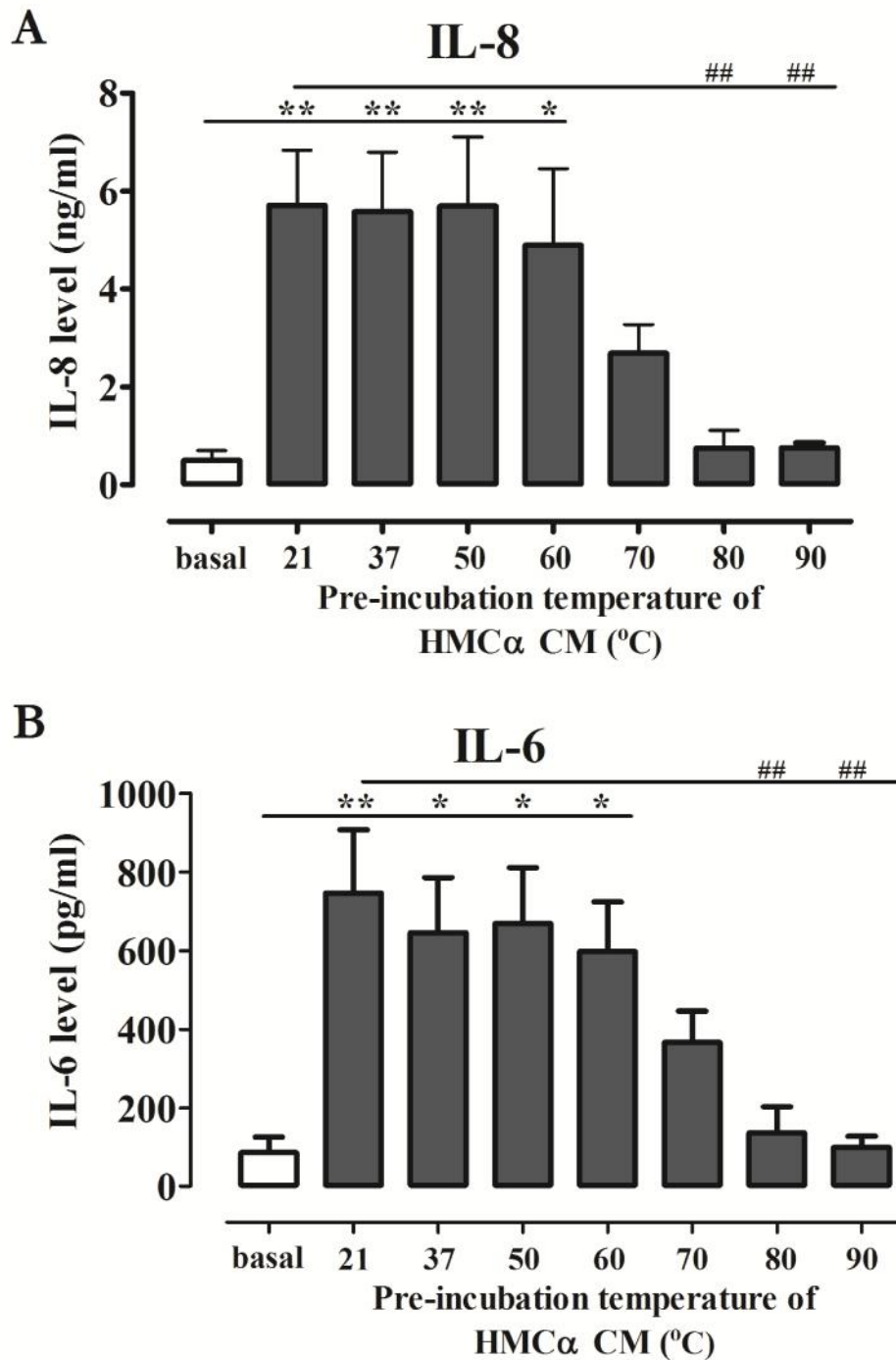
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Online suppl. fig. 1. HMC α cell-derived CM enhances cytokine mRNA levels in hASM cells. Following 24 hours serum-starvation, hASM cells were treated with/without non-stimulated HMC α cells CM (restCM) or antigen-stimulated HMC α cells CM (AgCM) for 6 or 16 hours. The mRNA levels of IL-8 (**A**) and IL-6 (**B**) were measured by qPCR and are expressed as a normalization of the threshold cycle numbers of the target gene versus 18S rRNA. Experiments were conducted on 3 separate experiments using 1 immortalized hASM cell line. Statistical differences were tested using two-way repeated measurement ANOVA, followed by Bonferroni's multiple post-hoc tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the control groups.



Online suppl. fig. 2. HMCα cell-derived active factor/s is/are susceptible to heat-inactivation. The hlgE/antigen-stimulated HMCα CM was pre-heated at different temperatures for 30 mins and then diluted 50-fold with fresh serum-depleted DMEM, prior to addition to hASM cells. Cell supernatants were harvested after 24 hours, IL-8 (**A**) and IL-6 (**B**) levels were measured. Results are expressed as the mean cytokine levels $\pm$ SEM of experiments conducted on 3 different hASM cell cultures. One-way repeated measures ANOVA, followed by Bonferroni's multiple post-hoc test. * $P < 0.05$, ** $P < 0.01$ compared to the basal release; ## $P < 0.01$ compared to HMCα CM incubated at 21°C.